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Immunocytochemical localization of α2,3(N)-sialyltransferase (ST3Gal III) in cell lines and rat kidney tissue sections: evidence for Golgi and post-Golgi localization

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Sialylation is a biosynthetic process occurring in the *trans* compartments of the Golgi apparatus. Corresponding evidence is based on localization and biochemical studies of α2,6(N)-sialyltransferase (ST6Gal I) as previously reported. Here we describe generation and characterization of polyclonal antibodies to recombinant rat α2,3(N)-sialyltransferase (ST3Gal III) expressed as a soluble enzyme in Sf9 cells or as a β-galactosidase-human-ST3Gal III fusion-protein from *E.coli*, respectively. These antibodies were used to localize ST3Gal III by immunofluorescence in various cell lines and rat kidney tissue sections. In transiently transfected COS cells the antibodies directed to soluble sialyltransferase or the sialyltransferase portion of the fusion-protein only recognized the recombinant antigen retained in the endoplasmic reticulum. However, an antibody fraction crossreactive with β-galactosidase recognized natively expressed ST3Gal III which was found to be colocalized with β1,4-galactosyltransferase in the Golgi apparatus of several cultured cell lines. Antibodies affinity purified on the β-galactosidase-ST3Gal III fusion-protein column derived from both antisera have then been used to localize the enzyme in perfusion-fixed rat kidney sections. We found strong staining of the Golgi apparatus of tubular epithelia and a brush-border-associated staining which colocalized with cytochemical staining of the H^+ATPase. This subcellular localization was not observed for ST6Gal I which localized to the Golgi apparatus. These data show colocalization in the Golgi apparatus and different post-Golgi distributions of the two sialyltransferases.

Key words: α2,3(N)-sialyltransferase/β-galactosidase/Golgi apparatus/kidney tubules/Jurkat cells

Introduction

Glycosyltransferases comprise a large group of enzymes involved in the synthesis of complex carbohydrates of glycoproteins, glycolipids and glycosaminoglycans. The enzymes are membrane-bound and localized predominantly to the Golgi apparatus and the *trans* Golgi network (TGN). In many cell types glycosyltransferases seem to be restricted to Golgi subcompartments (Roth, 1987). According to a widely accepted scheme, early acting Golgi glycosyltransferases are located in *cis* cisternae whereas late acting enzymes are mainly found in the *trans* Golgi or the TGN (for reviews, see Berger, 1985; Dunphy and Rothman, 1985; Farquhar, 1985). Galactose and sialic acid belong to the outer chains of N-linked carbohydrates; therefore, the respective enzymes galactosyl- and sialyltransferases are expected to be located in the *trans* Golgi and TGN. This location was confirmed by immunoelectron microscopy (Roth and Berger, 1982; Roth *et al.*, 1985) and by colocalization of β1,4-galactosyltransferase (β1,4-GT) with α2,6(N)-sialyltransferase (ST6Gal I) in conventional (Taatjes *et al.*, 1987) and confocal double immunofluorescence microscopy (Berger *et al.*, 1995). More recent reports suggested a less rigid confinement of glycosyltransferases since overlapping distributions of the different enzymes in the Golgi apparatus have been observed (Nilsson *et al.*, 1993; Rabouille *et al.*, 1995; for review, see also Rabouille and Nilsson, 1995). In addition, the distribution patterns of glycosyltransferases and processing enzymes seem to be cell-type specific (Roth *et al.*, 1986; Velasco *et al.*, 1993). Finally, there are reports about post-Golgi localizations of glycosyltransferases: While β1,4-GT has been postulated to be expressed at the cell surface since many years (Cooke and Shur, 1994), one report also showed a plasma membrane association of ST6Gal I by using protein-specific antibodies (Taatjes *et al.*, 1988). These findings raise the question whether glycosyltransferases are confined to their canonical Golgi localization or whether they can be targeted to post-Golgi sites. In the majority of cultured cell lines, glycosyltransferases are confined to the Golgi apparatus which appears as a compact juxtanuclear structure (Berger *et al.*, 1981). Cell surface expression of native glycosyltransferases in cultured cells are extremely uncommon; however, when transiently overexpressed in transfected cells they can be readily detected, as recently shown in the case of α1,3-fucosyltransferase V (Borsig *et al.*, 1996). Little is known on glycosyltransferase localization in tissue specimens. The histoarchitecture of tissues which affords an entirely different environment to the cells than growth in monolayers also modifies the shape of the Golgi apparatus (Taatjes *et al.*, 1987) and thereby may rearrange the localization of glycosyltransferases. Thus, localization studies conducted in tissue sections may unravel new sites that would not be anticipated from studies in cultured cells.

Since the introduction of cloning techniques to glycosyltransferase enzymology, powerful methods to delineate their organotypic expression are available such as Northern blots and *in situ* hybridization. However, Northern blots usually rely on crude RNA extracts from entire organs, which affords at best a rough orientation on the predominant expression sites. *In situ* hybridization may be useful to identify the cell types expressing a particular glycosyltransferase but would not address the question of its

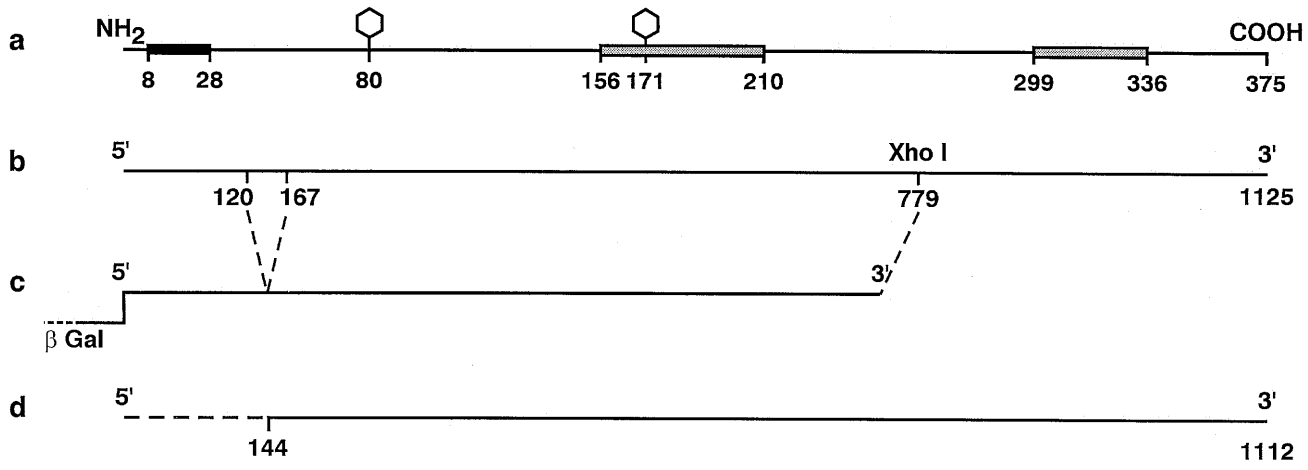

Fig. 1. Schematic view of ST3Gal III sequences. (**a**) Scheme of the rat ST3Gal III protein sequence with the cytoplasmic N-terminus; the transmembrane domain (dark bar); the luminal, catalytically active C-terminus; two conserved regions among sialyltransferases referred to as "sialyl-motifs" (gray bars) and two potential N-glycosylation sites (Kitagawa *et al.*, 1993). (**b**) Corresponding DNA sequence of ST3Gal III with the XhoI restriction site and the position of the observed deletion from bp 120 to 167. (**c**) ST3Gal III DNA sequence of the PCR-cloned form from Jurkat cells that was introduced into the expression vector which was used for the production of the β-galactosidase-ST3Gal III fusion-protein. (**d**) cDNA encoding truncated (broken line) soluble rat ST3Gal III used for expression in Sf9 cells using the baculovirus expression system.

subcellular localization. Thus, protein-specific antibodies for glycosyltransferases remain the tools of choice to address problems related to both tissular and cellular localization.

In this study we investigated the localization of α2,3(N)-sialyltransferase (ST3Gal III) by immunocyto- and -histochemical methods. Since mass expression and secretion of glycosyltransferases is well afforded by truncating them to soluble forms (Colley *et al.*, 1989), we used the powerful baculovirus-based expression system to obtain a pure soluble form of rat ST3Gal III to use as antigen (Gosselin *et al.*, 1994). In addition, we also used the strategy of expressing the glycosyltransferase antigen as a β-galactosidase fusion-protein in *E.coli* to obtain non-glycosylated peptide antigens for the induction of protein specific polyclonal antisera (Watzele *et al.*, 1991; Berger *et al.*, 1993) since presence of carbohydrate specific antibodies could cause misleading results (Feizi and Childs, 1987). The characterization of these newly developed antibodies led to the detection of an unexpected crossreactivity of antibodies directed against ST3Gal III with the β-galactosidase portion of the fusion-protein. We further present evidence that this phenomenon may be common and does not restrict specificity of the antiserum to the respective glycosyltransferase. We used the antibodies for immunohistochemistry to show evidence for Golgi and post-Golgi localization of ST3Gal III in rat kidney tubular epithelial cells.

Results

Antibodies to recombinant soluble rat ST3Gal III

A truncated form of rat ST3Gal III (see Figure 1) was expressed as a soluble recombinant enzyme in Sf9 cells using the baculovirus expression system (Gosselin *et al.*, 1994). The enzyme was purified on SP-Sepharose and CDP-ethanolamine-Sepharose to homogeneity and shown to exhibit a catalytic activity of 20 U/mg using Galβ1–3GlcNAcβ-O$(CH_2)_8$COOMe as an acceptor substrate. The enzyme was injected into rabbit 1 to induce a polyclonal antiserum (anti-rat ST3Gal III) as described in *Materials and methods*. The antiserum was tested by ELISA (Figure 2d) against soluble rat ST3Gal III (Figure 2d) as well as against human ST3Gal III-βgalactosidase fusion protein (not shown) and by immunofluorescence on COS-1 cells transfected with pcDNA-ST3Gal III. While only a low antiserum titer of 1:960 was measured, bright endoplasmic reticulum-associated fluorescence was detected in transiently transfected COS-1 cells (Figure 3b, inset). However, typical Golgi patterns were missing. From these data we concluded that (1) an antiserum was obtained specifically recognizing ST3Gal III, (2) the antiserum raised against a soluble rat enzyme crossreacted with its human homolog, and (3) that this truncated form essentially consisting of the catalytic domain was not very immunogenic. The absence of Golgi patterns in transfected cells could either be ascribed to poor transport competence out of the endoplasmic reticulum in COS cells or inability of the antiserum to recognize Golgi-associated forms. Nevertheless, using ST3Gal III fusion-protein affinity purified antibodies (anti-rat ST3Gal III *f*), we were able to detect weak staining of the Golgi apparatus in HepG2 cells (not shown). On rat tissue sections, however, purified anti-rat ST3Gal III *f* antibodies stained the Golgi apparatus in epithelial cells of proximal renal tubules and brush border-associated elements (not shown). Altogether, the fluorescent signals were considered too weak to warrant photodocumentation. These data supported findings reported previously that immunogenicity of glycosyltransferases is associated with the least conserved domain, i.e., the stem region (Watzele *et al.*, 1991; Berger *et al.*, 1993). Thus, we decided to raise antibodies to the full length enzyme as a β-galactosidase linked fusion-protein.

Construction of the α2,3(N)-sialyltransferase expression vector (pEX2-ST3Gal III) and expression of the β-galactosidase-α2,3(N)-sialyltransferase fusion-protein (ST3Gal III-FP)

Total RNA was isolated from Jurkat cells (human T cell line) and the mRNA fraction reverse transcribed to cDNA by oligo dT primers. The ST3Gal III sequence was amplified by PCR using specific primers flanking the full length of the coding region. Interestingly, Jurkat cells expressed two forms of ST3Gal III, one bearing a deletion of 48 bp from nucleotide position 120 to 167. Although this proved to be a finding which could be reproduced

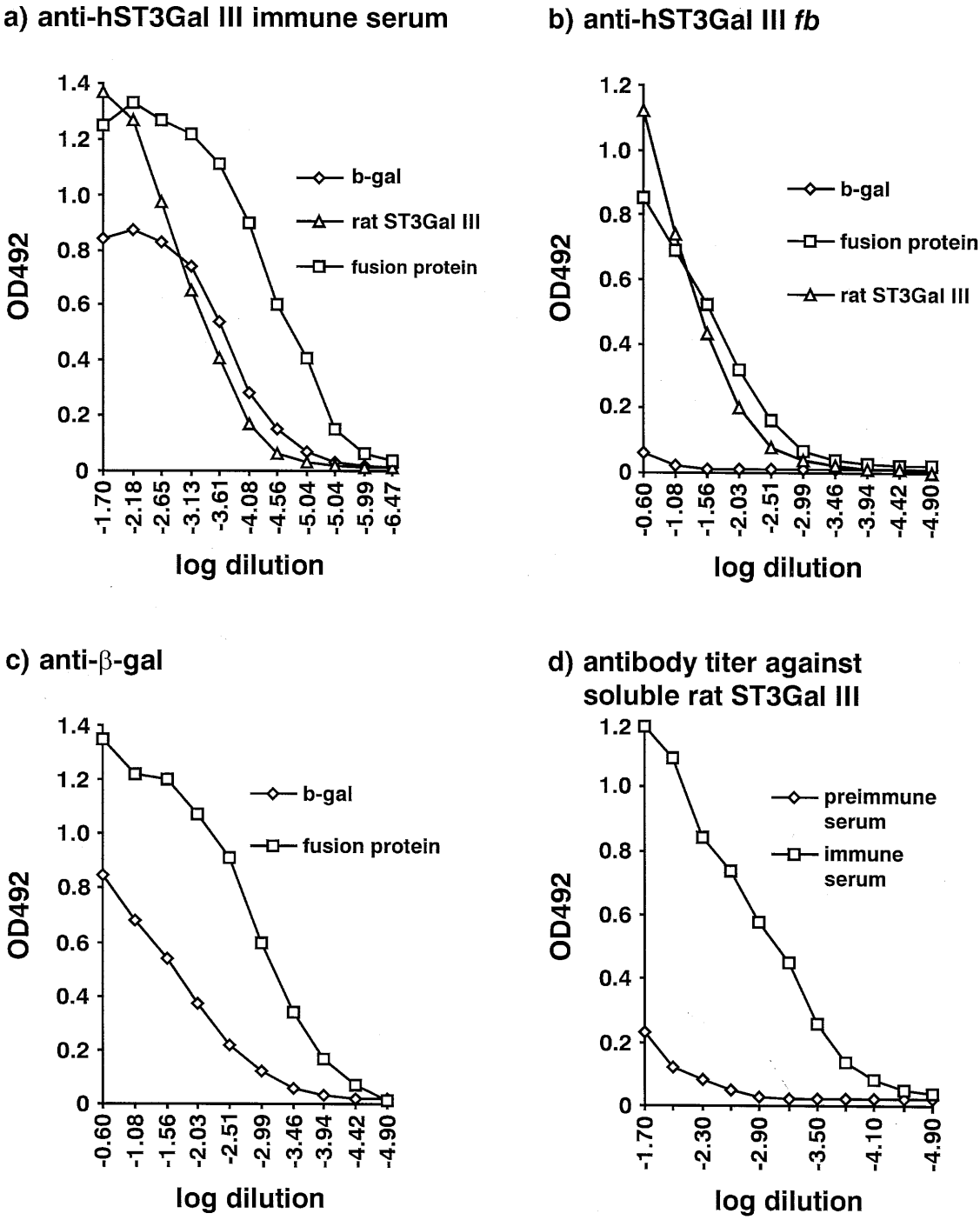

Fig. 2. Characterization of the different antibodies by ELISA. (a) Crossreactivity of anti-hST3Gal III immune serum with β-galactosidase (b-gal), truncated recombinant rat ST3Gal III (rat ST3Gal III) and β-galactosidase-ST3Gal III fusion-protein. (b) Crossreactivity of anti-hST3Gal III *fb* toward β-galactosidase, fusion-protein and rat ST3Gal III. (c) Crossreactivity of anti-β-*gal* towards β-galactosidase (b-gal) and fusion-protein. (d) Reactivity of anti soluble rat ST3Gal III immune- and preimmune serum toward soluble rat ST3Gal III.

from RNA extractions of several different cell lines we decided not to explore it further. The form of the ST3Gal III PCR product harboring the deletion was used for subsequent cloning into pUC18 vector. Sialyltransferases share two regions of homology (Sasaki *et al.*, 1993) which might lead to crossreacting antibodies. The ST3Gal III DNA sequence contains an internal XhoI restriction site (nucleotide position 779). XhoI was used in the subsequent cloning of ST3Gal III into the expression vector pEX2 to truncate the ST3Gal III DNA at its 3′ end. Thus, the sequence coding for one of the conserved regions was eliminated. Figure 1 shows (a) a scheme of the predicted full-length ST3Gal III protein from human placenta as reported by Kitagawa and Paulson (1993); (b) the corresponding DNA sequence; (c) the β-galactosidase-ST3Gal III DNA construct with the PCR-cloned

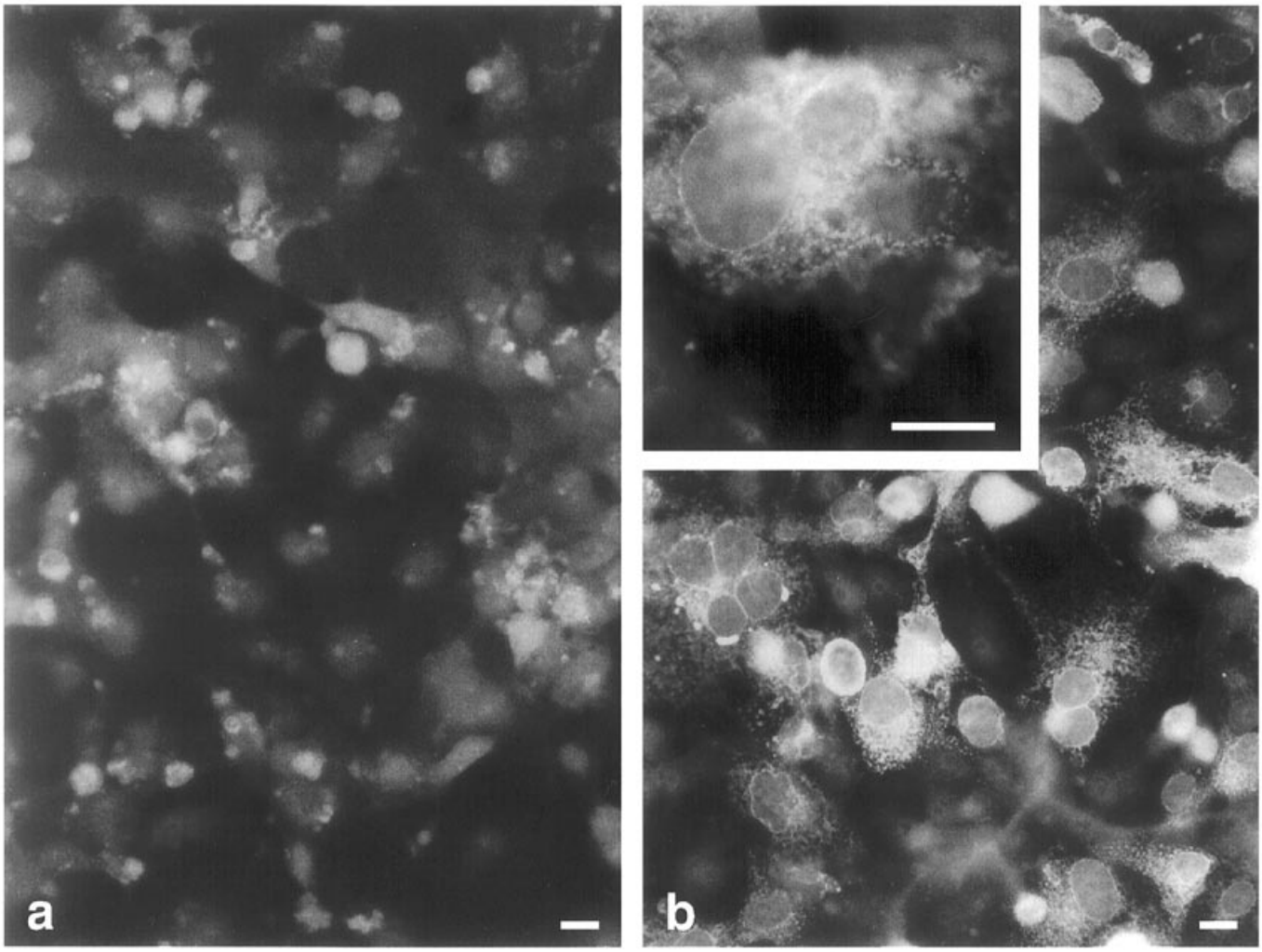

Fig. 3. Expression of human ST3Gal III in COS cells and immunodetection using an antiserum to recombinant soluble rat ST3Gal III. (a) Mock transfected COS cells stained with anti-rat ST3Gal III; (b) COS cells transiently transfected with pcDNA-ST3Gal III and stained with anti-rat ST3Gal III; transfected cells depict ER staining only (see inset). Scale bar, 20 μm.

form of ST3Gal III, harboring the 48 bp deletion, which led to the production of the antigen used for the immunization of rabbit 2; and (d) the truncated form of rat ST3Gal III used for expression in Sf9 cells.

The β-galactosidase ST3Gal III fusion-protein (ST3Gal III-FP) was produced in *E.coli* following transformation and induction. This heterologously expressed ST3Gal III-FP had a molecular weight of about 160 kDa. After separation of the proteins by SDS–PAGE, the band representing the fusion-protein was cut out, and the protein was eluted and analyzed for purity. Electrophoretically pure fractions (not shown) were then used for the immunization of rabbit 2 as described in *Materials and methods*. Another rabbit (rabbit 3) was immunized with a fusion-protein missing the first 29 N-terminal amino acids of ST3Gal III (but without deletion). Although specific, the antiserum failed to stain the enzyme in tissue cultured cells and was not used further.

Characterization of the antibodies against human ST3Gal III

The polyclonal antibodies against human ST3Gal III (anti-hST3Gal III) obtained by immunizing rabbit 2 with this heterologously expressed ST3Gal III-FP were characterized first by ELISA. The immune serum had a titer of about 1:8100 toward the coated ST3Gal III-FP, whereas binding of the preimmune serum (PIS) was negligible at the same dilution (not shown). The anti-hST3Gal III antiserum exhibited a lower titer towards purified β-galactosidase and the truncated recombinant form of rat ST3Gal III (Figure 2a). Since use of this antiserum to localize ST3Gal III in tissue specimens was intended, we prepared a fraction of affinity purified antibodies devoid of antibodies specific for β-galactosidase. The purification scheme (Figure 4) involved two affinity purification steps, first on the fusion-protein column (yielding anti-hST3Gal III *f*) followed by a β-galactosidase column (yielding anti-hST3Gal III *fb*). Antibodies retained on the β-galactosidase column were designated anti-β-*gal*. The corresponding binding curves are shown on Figure 2b and c and were as expected. As shown in Figure 5, these antibodies were tested by immunoblotting for reactivity toward ST3Gal III fusion-protein (slots 1, 4, 8), *E.coli* β-galactosidase (slots 2, 5, 7), and recombinant rat ST3Gal III (slots 3, 6, 9). The anti-hST3Gal III *f* (slots 1, 2, 3) reacted with all three antigens as predicted from the ELISA (Figure 2a). The anti-hST3Gal III *fb* (slots 7, 8, 9)

reacted with the fusion-protein (slot 8) and the rat ST3Gal III (slot 9), but not with β-galactosidase (slot 7), indicating efficient removal of these antibodies. Surprisingly, anti-β-*gal* antibodies (slots 4, 5, 6) also recognized the purified recombinant rat ST3Gal III (slot 6), indicating some sort of crossreactivity between β-galactosidase and the sialyltransferase portion of the fusion-protein. These results were confirmed by an independent method consisting of heterologous expression of the antigen (Jurkat cell-derived full length ST3Gal III) in COS cells followed by immunofluorescent staining of the expressed antigen. Figure 6 shows a panel of mock and pcDNA-ST3Gal III transfected COS cells. The anti-hST3Gal III *f* recognized endogenous Golgi-localized ST3Gal III in mock transfected COS cells (Figure 6a) and an overexpressed form also strongly decorating the Golgi apparatus (arrowhead) and ER-structures as indicated by the staining of the nuclear envelope (arrow, Figure 6c). To our surprise, removal of antibodies to β-galactosidase abrogated Golgi staining irrespective of its origin, i.e., both endogenous and transfected (Figure 6b), but this same anti-hST3Gal III *fb* fraction was able to recognize an ER-associated form of the transfected ST3Gal III (Figure 6d,e). Conversely, the antibodies to ST3Gal III eluted from the β-galactosidase column (anti-β-*gal* antibodies) were able to stain the Golgi apparatus (Figure 6f) in addition to some ER-associated forms (arrow). From these data we concluded that Golgi staining was conferred by ST3Gal III specific antibodies that crossreact with a common epitope also present on β-galactosidase. Immunoblots (Figure 5, slot 10) using the anti-hST3Gal III *fb* fraction on mock transfected COS cell lysate revealed one band at ~50 kDa, which may correspond to a mature and glycosylated, but denatured form of ST3Gal III since no Golgi staining was observed using this antibody fraction (Figure 6b). In ST3Gal III transfected COS cells (Figure 5, slot 11), the *fb* fraction identified additional forms at ~40 kDa. All these signals could be completely absorbed by addition of purified recombinant rat ST3Gal III (Figure 5, slot 12) while the fusion-protein was still recognized (slot 13). We attribute this reactivity to the recognition of denatured Golgi-associated forms of ST3Gal III (50 kDa) and not completely matured ER-associated forms (40 kDa). From these data we surmise that the antibody fraction *fb* specifically recognizes an epitope of ST3Gal III which may not be accessible in its native, Golgi-associated conformation. Conversely, those antibodies that crossreacted with β-galactosidase react with a conformation-independent epitope. This latter is likely to be associated with the N-terminus of ST3Gal III since a ST3Gal III construct from which the N-terminus was deleted was not able to induce an antiserum recognizing the Golgi apparatus but which also stained the ER of ST3Gal III-transfected COS cells (antiserum of rabbit 3, not shown).

We were then interested whether antisera previously raised to β-galactosidase fusion-proteins reacted in a similar way. We investigated two polyclonal antisera raised against a β-galactosidase-β1,4-GT fusion-protein (Watzele *et al.*, 1991) and a β-galactosidase-ST6Gal I fusion-protein (Berger *et al.*, 1993), respectively. Again, by purification on the β-galactosidase column we were able to isolate a β-galactosidase specific fraction of the antiserum to β1,4-GT fusion-protein which specifically stained the Golgi apparatus in HeLa cells; this staining could be completely blocked by addition of purified human milk β1,4-GT (not shown). Similarly, a β-galactosidase specific fraction of the antiserum to ST6Gal I fusion-protein also specifically stained the Golgi apparatus in HeLa cells (not shown). Dot blots then confirmed these findings and also showed that the antibodies to β-galactosidase crossreacted only with the glycosyltransferase portion of the fusion-protein against which they were raised but not with another one, e.g., the β-galactosidase specific fraction of the antiserum to β1,4-GT fusion protein did not crossreact with the β-galactosidase-ST6Gal I fusion-protein (not shown). Importantly, in the case of the antiserum to β1,4-GT fusion-protein, best Golgi staining was

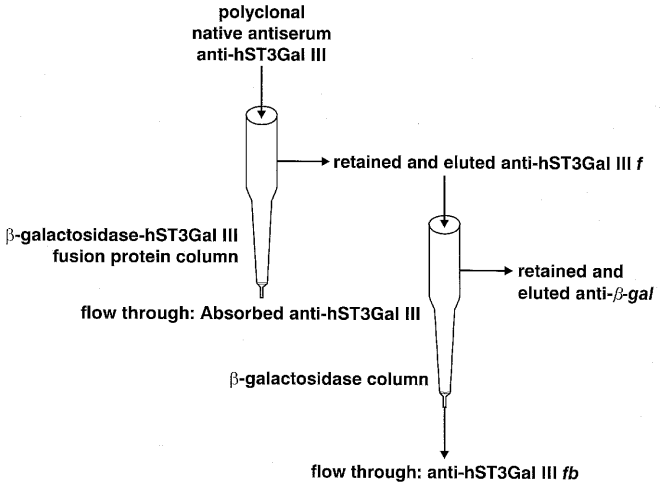

Fig. 4. Scheme of the affinity purification steps of anti-hST3Gal III antisera. Antibodies retained on the fusion-protein column (anti-hST3Gal III *f*) were subsequently affinity purified on a β-galactosidase column. The flow through of this column (anti-hST3Gal III *fb*) was specific for the ST3Gal III part of the fusion-protein. Antibodies retained on the β-galactosidase column (anti-β-*gal*) were also characterized.

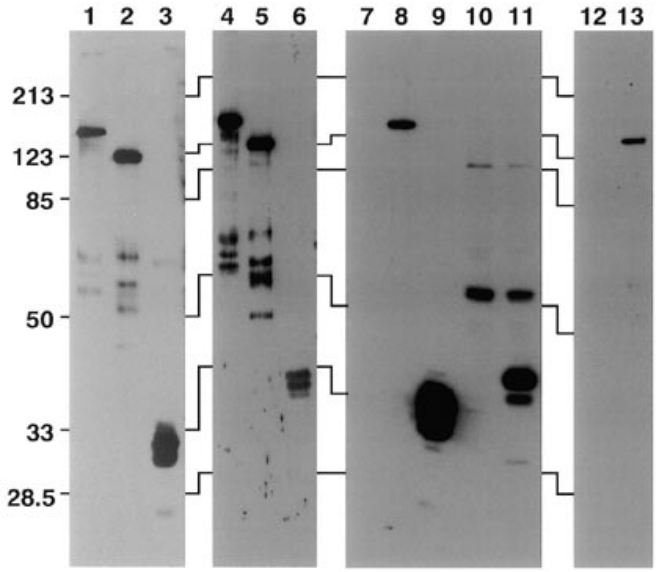

Fig. 5. Immunoblots using different antibody fractions. For technical details, see *Materials and methods*. Experiments from different blottings were aligned for the marker positions as indicated. Anti-hST3Gal III *f* (slots1, 2, 3), anti-β-*gal* (slots 4, 5, 6), and anti-hST3Gal III *fb* (slots 7, 8, 9, 10, 11) were probed for crossreactivity with fusion-protein (slots 1, 4, 8), β-galactosidase (2, 5, 7), rat ST3Gal III (3, 6, 9) and lysate of mock transfected (slot 10) and ST3Gal III-transfected (slot 11) COS cells. Anti-hST3Gal III *fb* was preabsorbed with rat ST3Gal III and tested for the remaining reactivity towards ST3Gal III-transfected COS cells (slot 12) and fusion-protein (slot 13).

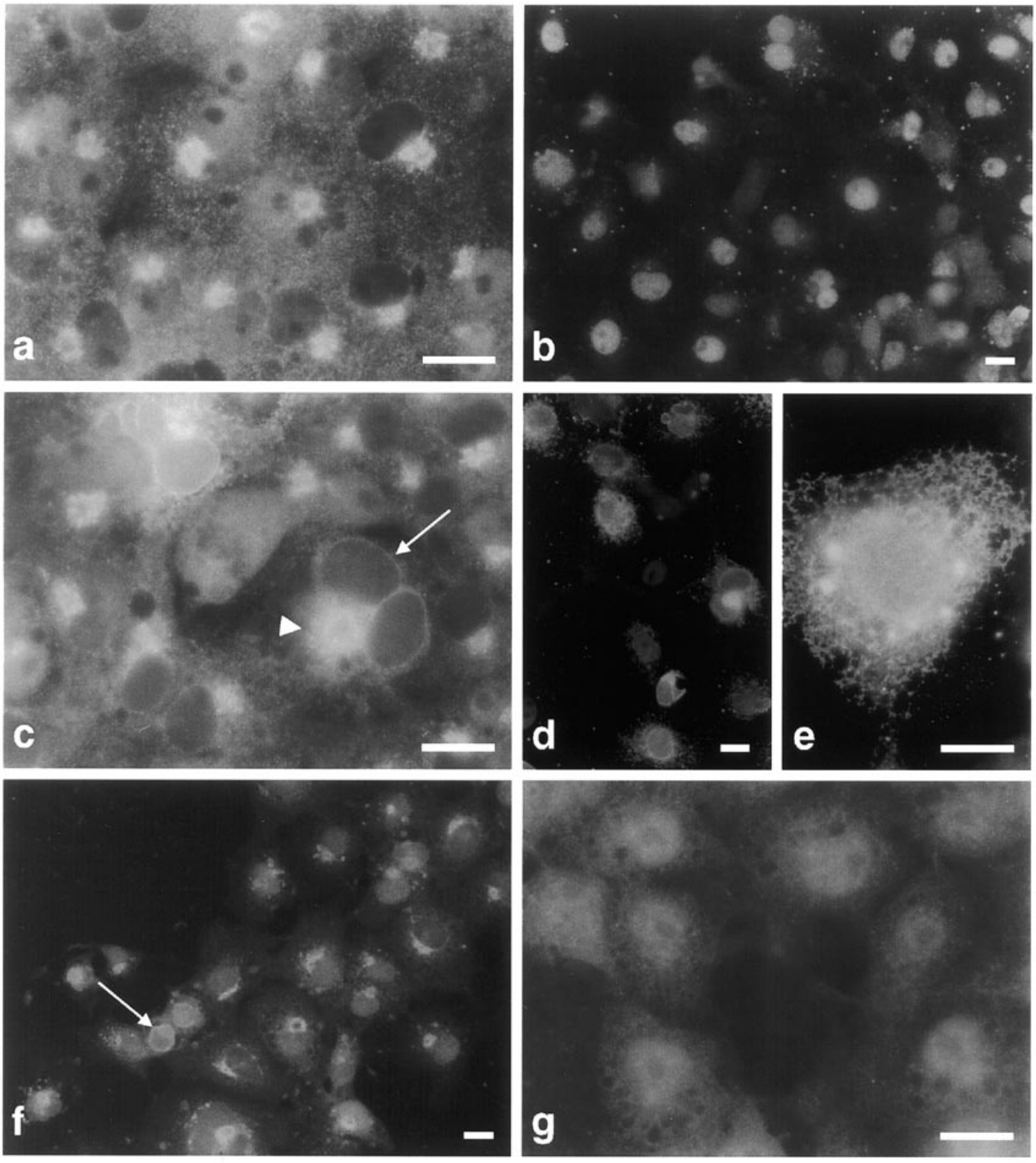

Fig. 6. Immunofluorescence microscopy of transiently transfected COS cells. (**a**) Mock transfected COS cells labeled with anti-hST3Gal III *f*. (**b**) Mock transfected COS cells labeled with anti-hST3Gal III *fb*; nuclear staining but no Golgi or ER signal was observed. The finding of nuclear staining was not further investigated. (**c**) ST3Gal III transfected COS cells labeled with anti-hST3Gal III *f*. This antibody recognized the overexpressed protein in an enlarged Golgi apparatus (arrowhead) and in the nuclear envelope (arrow) of the cells. (**d**, **e**) ST3Gal III transfected COS cells labeled with anti-hST3Gal III *fb*; only ER structures are stained. (**f**) ST3Gal III transfected cells labeled with anti-β-*gal*. Anti-β-galactosidase antibodies also recognized overexpressed ST3Gal III (arrow). (**g**) ST3Gal III transfected COS cells labeled with preimmune serum. Scale bar, 20 μm.

obtained with a fraction of anti-fusion-protein antiserum purified on immobilized human milk β1,4-GT (not shown). From these data we conclude that three antisera to three different β-galactosidase-glycosyltransferase fusion-proteins, respectively, contain a

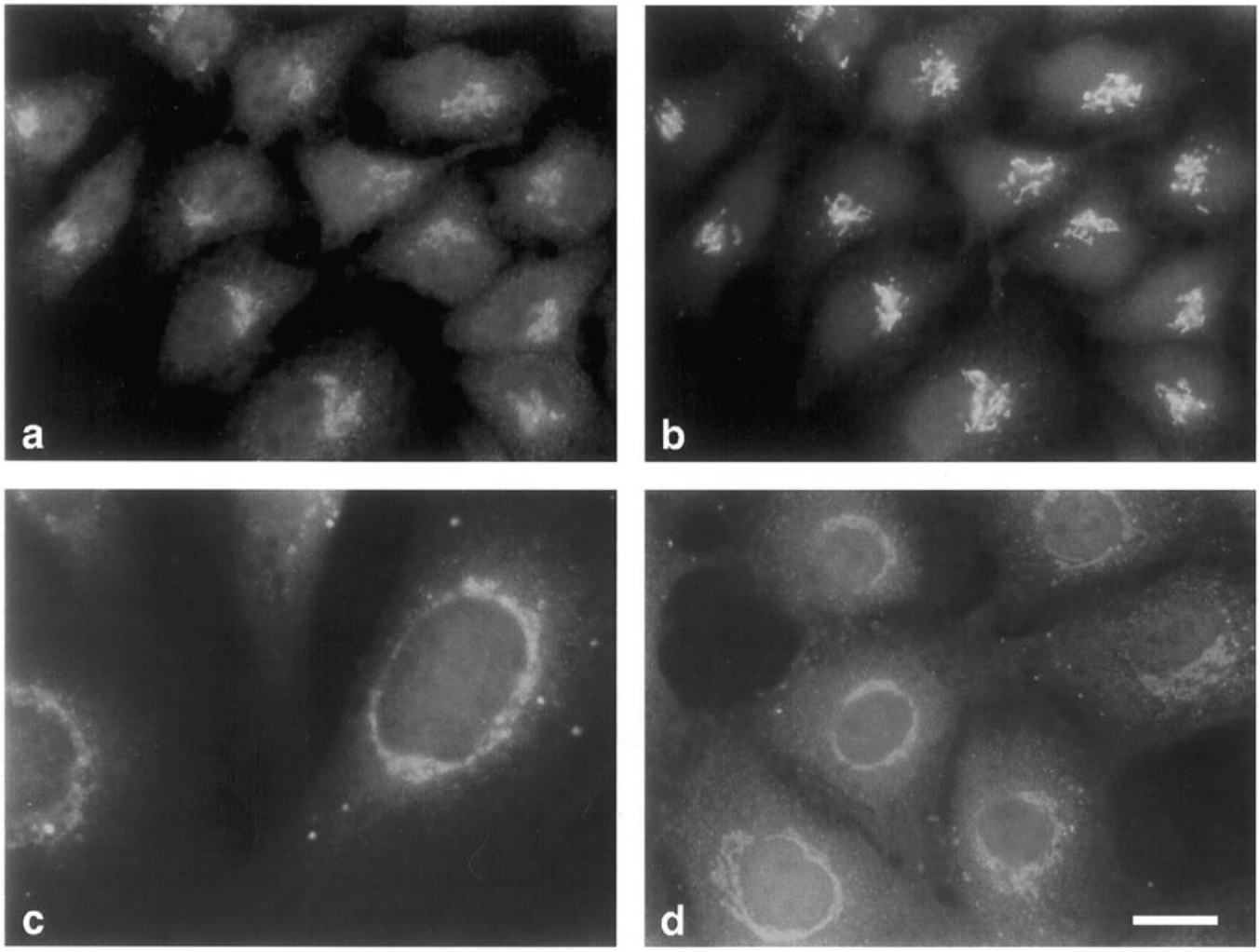

Fig. 7. Localization of ST3Gal III to the Golgi apparatus. Double immunofluorescence microscopy with (**a**) anti-hST3Gal III *f* and (**b**) monoclonal mouse antibody against β1,4-galactosyltransferase (mAb 36/118) for the localization of ST3Gal III in HeLa cells. Localization of ST3Gal III in (**c**) hamster CHO- and (**d**) rat IEC-cells. Scale bar, 20 μm.

fraction that crossreacted specifically with an epitope located on the β-galactosidase portion of the respective fusion-protein. Nevertheless these antibodies are specific reagents for the respective glycosyltransferase portion and thus considered suitable for immunohistochemical applications.

Application of anti-hST3Gal III antibodies for immunocytochemical localization in tissue cultured cells

ST3Gal III is localized in the Golgi apparatus of human HeLa cells as shown by colocalization of ST3Gal III with β1,4-GT by double immunofluorescence microscopy using the polyclonal anti-hST3Gal III *f* and the monoclonal anti-β1,4-GT antibodies (Berger *et al.*, 1986), respectively (Figure 7a,b). ST3Gal III colocalized with β1,4-GT also in other human cell lines, namely CaCo (colon adenocarcinoma), ECV304 (endothelial cell line), fibroblasts, and HepG2 (not shown).

Localization of ST3Gal III in rat kidney tissue

In immunofluorescence microscopy on cultured cells, anti-hST3Gal III *f* also stained the Golgi apparatus of hamster CHO (Figure 7c) and rat IEC cells (Figure 7d), which was not surprising since rat and human ST3Gal III share 97% homology on the amino acid level (Kitagawa and Paulson, 1993). The antibodies proved their usefulness for immunofluorescence microscopy on rat tissue sections. We performed localization studies of this glycosyltransferase in kidney since sialyltransferases are highly expressed in kidney as shown by Northern analysis (Wen *et al.*, 1992). ST3Gal III was localized to the Golgi apparatus of glomerular, tubular (Figure 8a), and interstitial cells. In addition, however, an unexpected location of ST3Gal III at the apical membrane of proximal convoluted tubules (PCT) and of single cells in connecting tubules (CNT) and collecting ducts (CD) was observed. Comparison of the apical ST3Gal III staining with the staining of H^+ATPase revealed on the one hand a striking similarity of localization of the enzyme and the proton pump at the brush border of PCT cells, and on the other hand the

confinement of apical ST3Gal III staining in CNT and CD to H^+ATPase-positive intercalated cells (Figure 8b). All of the observed staining was completely abolished by preabsorption of the antiserum with the respective antigen (Figure 8c), indicating that the staining was specific. Furthermore, control antisera that also contained antibodies to bacterial β-galactosidase did not stain, but the antiserum to recombinant soluble rat ST3Gal III produced identical results (not shown).

In order to localize ST3Gal III more precisely in the apical region of PCT, we combined the immunostaining of ST3Gal III with a staining of actin filaments using rhodamine-conjugated phalloidin (Figure 9a,b). The actin filament-rich microvilli were evenly stained by the phalloidin-conjugate, revealing them as morphologically intact. Within the brush border, ST3Gal III staining was strongest at the base of the microvilli, although a faint staining was detected along the entire microvilli. The same staining pattern was found also for H^+ATPase. Immunostaining of consecutive sections with anti-hST3Gal III serum and a specific antibody to rat ST6Gal I (Bosshart and Berger, 1992), respectively, revealed a clear Golgi localization of both sialyltransferases (Figure 9c,d). In contrast to ST3Gal III, no significant apical expression of ST6Gal I was detected.

Discussion

In this report, we describe production and characterization of antibodies to recombinant human Gal-β1,3(4) GlcNAc α2,3(N)-sialyltransferase (ST3Gal III) by different biochemical and cell biological methods. ST3Gal III shares two regions of homology with other sialyltransferases (Wen *et al.*, 1992; Sasaki *et al.*, 1993; Datta *et al.*, 1995). These two peptide stretches, also called sialylmotifs (see Figure 1), were found on all the sialyltransferases cloned so far and seem to be characteristic for the sialyltransferase family. These sialylmotifs are potential crossreactive epitopes for our antibodies. One of these sialylmotifs is located at the C-terminus of ST3Gal III. To eliminate this C-terminal part in the recombinant protein, we took advantage of an internal XhoI restriction site in the ST3Gal III nucleotide sequence and used a C-terminal truncated form for the construction of the ST3Gal III expression vector. The other sialylmotif seemed not to be recognized by our anti-hST3Gal III antibodies. Whereas preincubation of the anti-hST3Gal III antibodies with the corresponding antigen (ST3Gal III-FP) completely abolished a specific immunofluorescent signal, preincubation with another sialyltransferase fusion-protein (ST6Gal I fusion-protein; Berger *et al.*, 1993), bearing the respective sialylmotif, did not alter binding of the antibodies (not shown). These results indicate that antibodies to ST3Gal III do not crossreact with ST6Gal I and can thus be considered monospecific. The hitherto known and cloned sialyltransferases have recently been reviewed: except for the presence of the sialylmotifs little homology has been detected (Harduin-Lepers *et al.*, 1995). Thus, crossreactivities among different sialyltransferases are not likely to occur since the sialylmotif did not appear to be antigenic whereas interspecies crossreactivity was strong since on the amino acid level, ST3Gal III is conserved to a degree of 97% between rat and human (Kitagawa and Paulson, 1993). Weinstein and colleagues purified the homologous enzyme from rat liver to homogeneity (Weinstein *et al.*, 1982) and obtained a single band with a molecular weight of 44 kDa. By immunoblotting of COS cell lysates (Figure 5), our antibodies detected a single band at around 50 kDa. Unglycosylated ST3Gal III has a theoretical molecular weight of about 43–44 kDa with two potential N-glycosylation sites. The observed difference in the molecular weight may be due to different glycosylation by COS cells and the possibility that the form identified by Weinstein *et al.* might have been processed.

Since the homology of ST3Gal III among different species seems to be quite high, it was not surprising that the antibodies showed crossreactivity with the corresponding enzymes of rat IEC-, mouse L-, and simian COS-1 cells. In all the tested human cell lines, ST3Gal III colocalized with galactosyltransferase, indicating that this enzyme, like ST6Gal I (Roth *et al.*, 1985), is located in the *trans* Golgi/TGN of these cells. Independent evidence for colocalization of both α2,3- and α2,6(N)-sialyltransferase has been obtained by transfection of the latter in CHO cells; thereby, the linkages of terminal sialic acid residues were changed partially from 2–3 to 2–6 positions (Lee *et al.*, 1989).

Unexpected, however, was the observation made by immunofluorescence microscopy on rat kidney tissue. In the proximal convoluted tubule and in intercalated cells of the connecting tubule and the collecting duct, ST3Gal III was localized in the Golgi and additionally in the region of the apical membrane (Figure 8). This staining corresponds to a bona fide localization of ST3Gal III since conventional controls accepted in immunocytochemistry such as abolition of staining by absorption with antigen support this assertion. Moreover, use of another antiserum raised against a different form of the enzyme (soluble, Sf9 cell expressed rat enzyme) produced identical staining patterns. In addition, crossreactivity with carbohydrate antigens is very unlikely to occur with antibodies against peptide antigens. We were interested in assessing the possibility that antibodies to β-galactosidase present in our antibody preparations could crossreact with an unrelated protein. Although this possibility could not formally been excluded, we presented evidence that those antibodies which specifically decorated the Golgi apparatus reacted there with ST3Gal III but also crossreacted with β-galactosidase, which could be due to a common epitope in the link region from β-galactosidase to ST3Gal III.

At the light microscopic level the exact nature of the apical structures positive for the presence of ST3Gal III (cell surface or subapical vesicles) can not be clearly defined. In the proximal tubule almost complete colocalization at the light microscopic level of ST3Gal III with H^+ATPase, a protein known to be located in the brush border membrane (Brown *et al.*, 1988) as well as in subapical vesicles suggest the occurrence of ST3Gal III at both locations. This post-Golgi localization for a sialyltransferase is not without precedent: Taatjes and colleagues reported the localization of ST6Gal I in post-Golgi vesicles, brush border and basolateral membranes, multivesicular bodies and lysosome-like structures of absorptive enterocytes. In addition they found ST6Gal I in mature mucus droplets and in the plasma membrane of goblet cells (Taatjes *et al.*, 1988). Interestingly, in kidney tubular epithelial cells ST6Gal I did not colocalize at this post-Golgi site with ST3Gal III indicating a sorting mechanism between these two glycosyltransferases.

Brada reported a similar unexpected location of glucosidase II in pig kidney epithelial cells (Brada *et al.*, 1990): Glucosidase II previously known as a resident ER enzyme, was found as a sialoglycoprotein predominantly in post-Golgi compartments of proximal tubules similar to those described here for the presence of ST3Gal III. Also in this case, the functional implication of such a localization remained obscure.

The luminal cell surface of kidney proximal tubular cells, similar as in intercalated cells, is highly glycosylated and is subjected to a very high turnover (for review, see Brown, 1989). Sialyltransferases in the region of the luminal membrane might

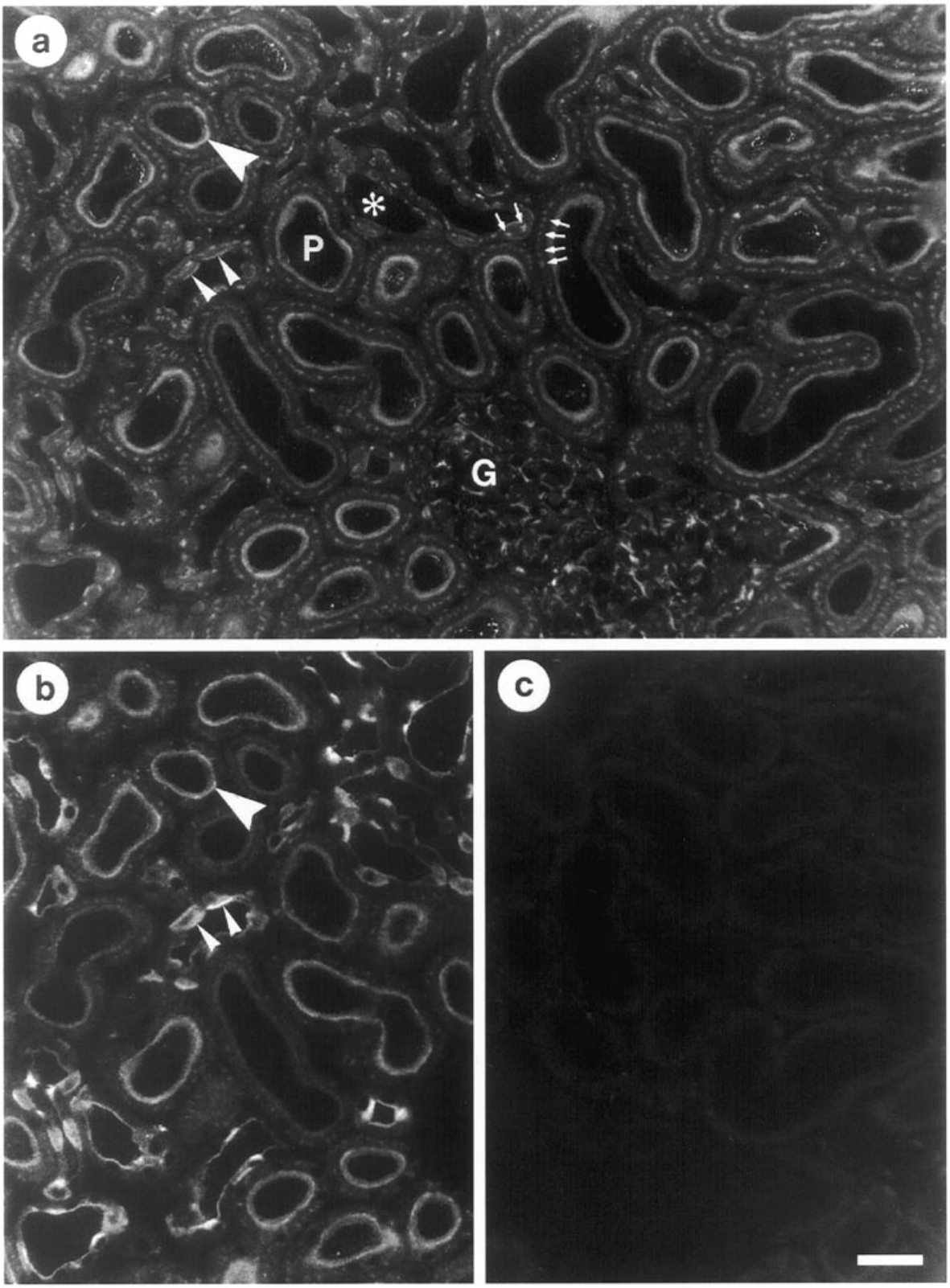

Fig. 8. Overview of immunolocalization of ST3Gal III on serial cryosections or rat kidney. (**a**) Immunohistochemistry with affinity purified anti-hST3Gal III antiserum (fraction antiST3Gal III *f*; see text) produces a staining in epithelial cells of proximal tubules (P) and connecting tubules (asterisk) in some cells of the glomeruli (G) and in interstitial cells. In epithelial cells, an intracellular staining at the typical perinuclear Golgi-localization can be recognized (small arrows). Luminal staining of epithelial cells is also detected (arrowheads). (**b**) Luminal ST3Gal III can be partially colocalized with H^+ATPase which is found in proximal tubular cells at the base of the apical brush border (large arrowhead) and in intercalated cells (small arrowheads). (**c**) Absorption with ST3Gal III-β-galactosidase fusion protein of anti-hST3Gal III antiserum completely prevented immunostaining of a consecutive kidney section. Scale bar, 50 μm.

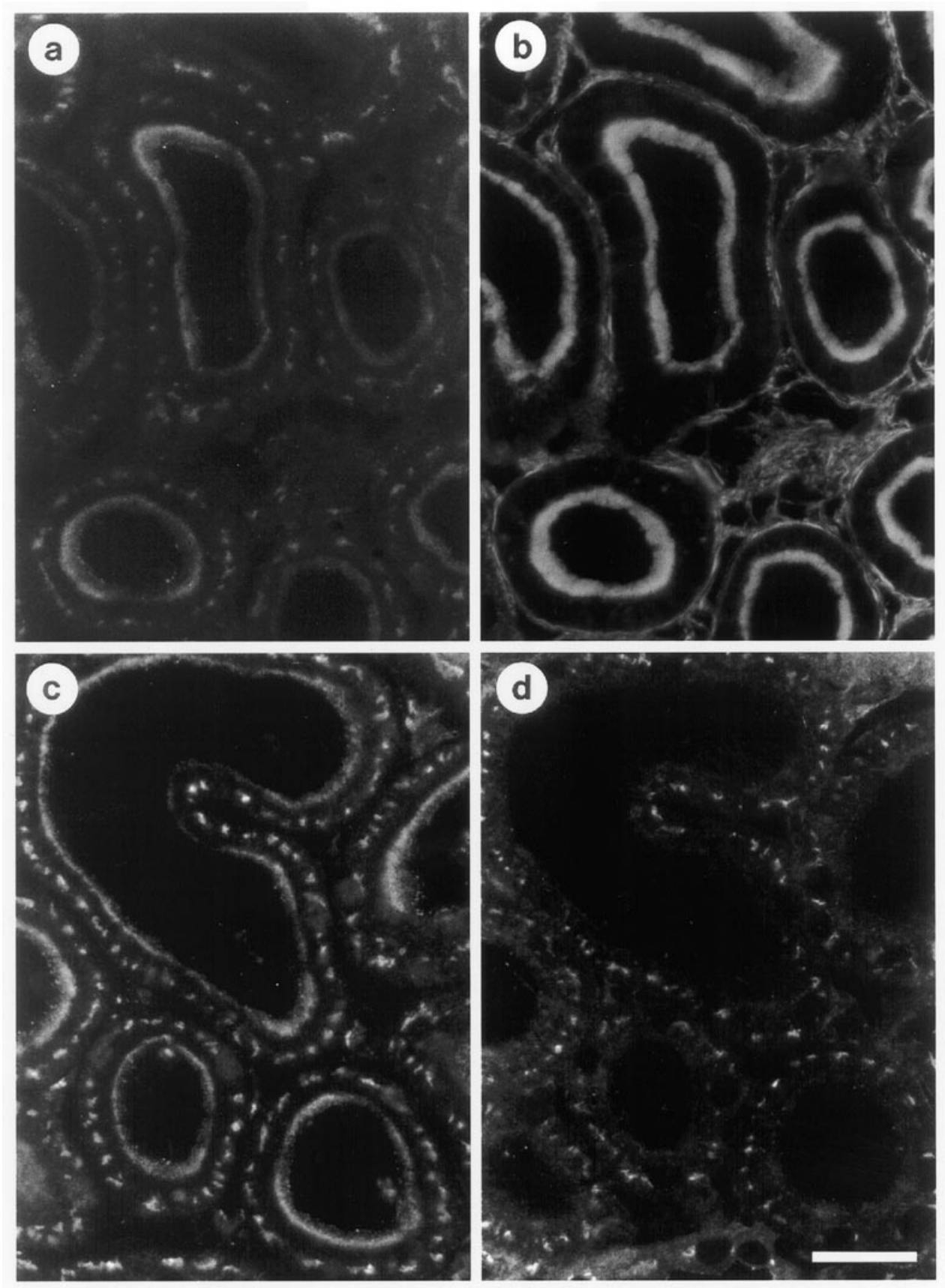

Fig. 9. Comparison of the proximal tubular localization of ST3Gal III with actin filaments and of ST3Gal III with ST6Gal I. (**a**, **c**) The ST3Gal III specific antiserum decorates intracellularly a juxtanuclear (Golgi) region and apically the brush border where staining appears strongest at the base. (**b**) Co-staining of the section in (**a**) for actin filaments depicts the brush border in their full width. (**d**) Staining of a section consecutive to (**c**) for ST6Gal I reveals a similar Golgi localization of the enzyme and its absence in the apical region. Scale bar, 50 μm.

have a function in the resialylation of recycling cell surface glycoproteins. Such a resialylation process in the endocytotic compartment has been well documented in cultured cells (Snider and Rogers, 1985; Duncan and Kornfeld, 1988). Further work is needed to define the exact localization of ST3Gal III at these post-Golgi sites and to identify any corresponding functional implications.

Materials and methods

Materials

Polyclonal antibodies to rat ST6Gal I (Bosshart and Berger, 1992) and monoclonal antibodies to β1,4-GT (Berger *et al.*, 1986) were described previously. Monoclonal antibodies to H^+-ATPase were kindly provided by S. Gluck (Hemken *et al.*, 1991).The origins of commercially available material are indicated below. All cell lines used were from ATCC (Rockville, MD). Chemicals were of analytical grade purity and obtained from Fluka (Buchs, Switzerland).

Construction of α2,3N-sialyltransferase vectors

Rat α2,3(N)-sialyltransferase. A soluble and exported form of the α2,3(N)-sialyltransferase from rat liver was cloned in baculovirus as described previously by Gosselin *et al.* (1994).

Human α2,3(N)-sialyltransferase.Total RNA from the Jurkat cell line (T cell line) was isolated by the RNAgent kit (Promega, Madison, WI). Double stranded cDNA was synthesized by reverse transcription using oligo dT primers synthesized by Microsynth (Windisch, Switzerland) and Superscript RT polymerase from Gibco (Grand Island, NY). Oligonucleotides (Microsynth) corresponding to the flanking regions of the full length of the ST3Gal III sequence were used as specific primers for PCR amplification ("ST3Gal III/Nco.up": 5′-ggccATGGGACTCTTGGTATTTGT-3′ and "ST3Gal III/Xho.down": 5′-gcctcgagTCAGATGCCACTGCTTA-3′). These primers contained at their 5′ ends additional consensus sequences for the restriction enzymes NcoI and XhoI (small letters), respectively. The PCR procedure conditions were 95°C for 40 sec, 55°C for 55 s, and 72°C for 90 s for denaturation, annealing and polymerization, respectively. The obtained 1.1 kb band was excised from the 1% low melt agarose gel and used for another round of amplification under the same conditions. After the second round, the DNA in the excised band was isolated from the gel by the Geneclean II kit (BIO 101 Inc., La Jolla, CA). This cDNA was treated with Klenow Fragment (Promega, Madison, WI) and ligated into SmaI (Promega) cleaved vector pUC18 (pUC18-ST3Gal III). This plasmid was transfected into competent XL1-blue cells (Stratagene, La Jolla, CA). Since ST3Gal III contains an internal XhoI restriction site (nucleotide position 779), we used the 3′ end (C-terminal) truncated form of double digested NcoI/XhoI pUC18-ST3Gal III for subcloning into SmaI cleaved expression vector pEX2 (Clontech, Palo Alto, CA). Inframe cloning of ST3Gal III to the β-galactosidase sequence of the expression vector as a β-galactosidase-α2,3(N)-sialyltransferase fusion-protein (pEX-ST3Gal III-FP) was checked by sequencing with the T7 sequencing Kit from Pharmacia (Uppsala, Sweden).

For the expression of ST3Gal III in COS-7 cells a completely sequenced, full length PCR product was cloned into EcoRV cleaved pcDNAI (Invitrogen, San Diego CA) further referred to as pcDNAI-ST3Gal III.

Production and purification of soluble recombinant rat ST3Gal III

Recombinant baculovirus was used to infect Sf9 cells, grown at 28°C in EX-CELL 400 medium (JHR Biosciences) supplemented with 5% FCS to a cell density of 2×10^6. The multiplicity of infection was about 20. Sixty hours postinfection the conditioned medium was spun, filtered through 0.8 μm membrane, and 10-fold concentrated using a spiral ultrafiltration cartridge (YM30, Amicon). All further purification steps were carried out at 4°C. The enzyme solution was loaded onto a SP-Sepharose column (Fast-flow, Pharmacia), equilibrated with buffer A (50 mM Na cacodylate, pH 6.5). The column was washed with buffer A and eluted with buffer A containing 500 mM NaCl, 20% glycerol (v/v). The eluate was diluted with buffer A to 150 mM NaCl, 6% glycerol and loaded onto a CDP-ethanolamine-Sepharose affinity column, equilibrated with buffer A containing 150 mM NaCl. After washing the column with equilibration buffer, the enzyme was eluted with buffer A containing 1 M NaCl, 1 mg/ml CDP. The eluate was brought to 50 mM Na cacodylate, pH 6.5, 250 mM NaCl, 250 μg/ml CDP. Aliquots of the enzyme (6 U/ml) were stored at -80 °C.

Production and purification of ST3Gal III-FP

Plasmid pEX2-ST3Gal III-FP was transformed into the temperature sensitive *E.coli* strain N4830–1 (Clontech). After induction of protein expression by temperature shift, cells were harvested by centrifugation and lysed, and their inclusion bodies, containing the ST3Gal III-FP were purified as described previously (Berger *et al.*, 1993). ST3Gal III-FP was obtained by fractionation of the inclusion bodies on a 7.5% SDS–polyacrylamide gel, excision of the ST3Gal III-FP band from the gel and elution of the fusion-protein using the biotrap from Schleicher & Schuell (Dassel, D) with 15 mM NH_4HCO_3 buffer.

Induction of antibodies and their purification.

Antiserum to soluble rat ST3Gal III. Purified antigen (62.5) μg of emulsified with complete Freund adjuvant (Sigma, St. Louis, MO/USA) was subcutaneously injected into rabbit 1 (New Zealand White) followed by five boost injections with incomplete adjuvant every fortnight.

Antiserum to human ST3Gal III-FP. An analogous scheme to immunize rabbits 2 and 3 was applied as for the rat ST3Gal III, except that 108 μg fusion-protein was injected at a time.

The third rabbit was injected a modified form of the fusion-protein (see results). For construction of the affinity columns 0.8 mg of ST3Gal III-FP and 0.9 mg of β-galactosidase (Sigma) were immobilized on Affi-Gel 10 and 15 (Bio-Rad, Richmond, CA), respectively, according to the user's manual. The ST3Gal III-FP column was equilibrated with buffer H (100 mM HEPES, pH 7.9) at 4°C. All three antisera were purified on the ST3Gal III-FP column by loading them five fold diluted in the same buffer H. After shaking overnight, the column was washed with buffer H containing 500 mM NaCl. Bound antibodies were eluted with 100 mM glycine-HCl pH 2.8, immediately neutralized in 1 M HEPES pH 10, and dialyzed against Tris buffered saline (TBS). The following designations are used. On the ST3Gal III-FP column affinity purified antibodies derived from antiserum to recombinant soluble rat ST3Gal III: anti-rat ST3Gal III *f*; from antiserum to ST3Gal III-FP: anti-hST3Gal III *f*; also see scheme (Figure 1). An antibody fraction of anti-hST3Gal III *f* was then loaded on a β-galactosidase column that was previously equilibrated with

TBS. After shaking overnight at 4°C the flow through of this column was collected (anti-hST3Gal III *fb*) and concentrated in a 100 kDa cut-off Centricon (Amicon, Beverly, MA/USA). After washing the β-galactosidase column with TBS/500 mM NaCl, retained antibodies (anti-β-*gal*) were eluted with 100 mM glycine-HCl pH 2.8, immediately neutralized in 1 M Tris pH 10, dialyzed against TBS, and concentrated with Centricon 100. Figure 1 shows a scheme of the different fractions of antibodies to ST3Gal III.

ELISA

Immunoplates (Nunc Maxisorp, Gibco) were coated overnight at 4°C with 0.37 µg fusion-protein, 0.4 µg β-galactosidase and 1.2 µg rat ST3Gal III. The protein concentration was inversely related to the length of the corresponding protein to match for the number of epitopes. The following day, antibodies diluted in 100 mM NaCl, 0.05% Tween 20 were incubated in the indicated dilutions for 90 min at room temperature. After two washes with NaCl/Tween 20, 1:2000 diluted horseradish peroxidase (HRP) coupled with goat anti-rabbit IgG (Bio-Rad) was incubated for 90 min. After two final washes with NaCl/Tween 20, substrate solution containing 50 mM Na_2HPO_4, 25 mM citric acid, 0.12% (v/v) H_2O_2, and 0.04% (w/v) 1,2-phenylenediamine was added. The reaction was stopped by the addition of 1.25 M H_2SO_4. Optical density was measured in an ELISA reader at 492 nm.

Transfection of COS cells

COS-7 or COS-1 cells were grown to 50% subconfluency either in 100 mm dishes or for immunofluorescence microscopy on glass cover slips in 33 mm dishes. The cells were transfected for transient protein expression with 3 µg (100 mm) and 0.5 µg (33 mm) plasmid pcDNAI-ST3Gal III (=ST3Gal III transfected cells) or pcDNAI alone (=mock transfected cells) using lipofectin (LipofectAMIN from Gibco) as indicated by the manufacturer. After 48 h cells were lysed for immunoblots or treated as described below.

Immunofluorescence microscopy on cultured cells

Cells were cultured at 37°C on glass coverslips to subconfluency. Before staining, they were washed with phosphate-buffered saline (PBS), fixed for 10 min by prewarmed (37°C) 3.5% (w/v) paraformaldehyde in Hanks Balanced Salt Solution (GibcoBRL). All further steps were carried out at room temperature. Cells were rinsed three times with PBS, quenched for 10 min with 20 mM glycine in PBS, rinsed again three times with PBS, and permeabilized for 30 min by 0.1% (w/v) saponin in PBS (sap/PBS). The permeabilized cells were then incubated for 1 h at room temperature with suitably diluted first antibodies, washed three times with sap/PBS, and covered 1 h at room temperature with 50 fold diluted Texas red (TR) conjugated goat anti rabbit antibodies (Organon Teknika–Cappel, Turnhout, B) or fluorescein-isothiocyanate (FITC) conjugated goat anti-mouse antibodies (Dako). After three washes with sap/PBS the coverslips were mounted on glass slides with Mowiol-N-propyl-gallate (Berger *et al.*, 1993).

Immunoblot analysis

Subconfluent HeLa and transfected COS cells from 100 mm petri dishes were harvested and lysed during 45 min at 4°C in PBS containing 1.5% Triton X-100 and 1 mM phenylmethansulfonyl-fluoride (PMSF). Lysates were cleared by centrifugation for 15 min at 15,000 × *g*. For immunoblot analysis, proteins from lysates and purified proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on a 10% gel and electrotransferred to a nitrocellulose membrane. The membrane was blocked with blocking buffer (1% milk powder, 15 mM NaCl, 5 mM Tris, 0.1% NP-40 pH 7.5) at 4°C overnight. The blot was then probed 1 h with suitably diluted first antibodies, washed three times 20 min with blocking buffer, incubated 1 h with horseradish peroxidase (HRP)-conjugated anti rabbit Ig G (Sigma) 1:10,000 diluted in blocking buffer, and washed again three times with blocking buffer. The blot was developed with the "enhanced chemiluminescence (ECL) detection kit" (Amersham, Buckinghamshire, UK).

Immunohistochemistry on rat kidney

Rats (Sprague Dawley) were anesthesized with thiopental 100 mg/kg body weight, injected intraperitoneally, and perfused retrogradely at a pressure of 1.38 hp through the abdominal aorta with a fixative of 3% paraformaldehyde and 0.05% picrinic acid in a 6:4 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mM with sucrose) and 10% hydroxyethyl starch (HAES steril). After 5 min of fixation, the fixative was replaced by perfusion for 5 min with cacodylate buffer.

Slices of fixed kidneys were frozen in liquid propane and cooled by liquid nitrogen. Sections of 3 µm were cut at -22°C in the cryomicrotome, mounted on chromalum/gelatine-coated glass slides, thawed, and stored in cold PBS until use.

For immunofluorescence staining, sections were preincubated for 10 min with 3% milk powder in PBS containing 0.3% Triton X-100. They were then covered overnight at 4°C with primary antibodies diluted as indicated below. The sections were rinsed three times prior to incubation for 1 h at 4°C with secondary antibodies and/or rhodamine-conjugated phalloidin. All immunochemicals were diluted in PBS/3% milk powder. Dilutions were 1:500 for the anti-hST3Gal III *f* antiserum, 1:20 for the anti-H^+ATPase antibody, 1:50 for swine anti rabbit IgG conjugated to FITC (Dakopatts, Glostrup, Denmark), 1:200 for goat anti-mouse IgG conjugated to Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA), and 1:100 for phalloidin-rhodamine (Molecular Probes Inc., Eugene, OR). After being rinsed with water the sections were coverslipped using DAKO-Glycergel (Dakopatts) plus 2.5% 1,4-diazabicyclo{2.2.2}octane (Sigma) as a fading retardant. All immunofluorescence preparations were studied with a Polyvar (Reichert-Jung) microscope equipped for epifluorescence and using narrow-band filter systems for FITC and Cy3.

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Abbreviations

β1,4-GT, UDP-Gal:GlcNAcβ1-R β1,4-galactosyltransferase (E.C. 2.4.1.38); ST3Gal III, CMP-sialic acid:Galβ1–3/4GlcNAc-R α2,3-sialyltransferase (E.C. 2.4.99.6); ST6Gal I, CMP-sialic acid:Galβ1–4GlcNAc-R α2,6-sialyltransferase (E.C. 2.4.99.1);

ST3Gal III-FP, ST3Gal III fusion-protein; pcDNA-ST3Gal III, pcDNA vector containing full length human ST3Gal III cloned from the Jurkat cell line.

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